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REGULAR ARTICLE

Derivation, characterization, and gene expression profile of two new human ES cell lines from India

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Abstract Human embryonic stem cells (hESCs) offer new avenues for studying human development and disease progression in addition to their tremendous potential toward development of cell-replacement therapies for various cellular disorders. We have earlier reported the derivation and characterization of Relicell[®] hES1, the first fully characterized hESC line generated from the Indian subcontinent. Recent studies have demonstrated discrete differences among hESC lines, in terms of both their growth properties and their differentiation propensity. To address some of these issues in the context of hESC research in India, we have recently generated two new hESC lines: Relicell[®] hES2 and Relicell[®] hES3. Both these cell lines were derived using a combinatorial approach of immunosurgery followed by mechanical surgery for inner cell mass isolation. The cell lines exhibit the usual hESC characteristics including their ability to differentiate both *in vitro* and *in vivo* to yield the three germinal layers. Whole genome microarray analysis of these cell lines was compared with Relicell[®] hES1 and it showed that approximately 9000 genes were expressed by these lines. As expected the expression pattern of these new cell lines bore close resemblance to that of Relicell[®] hES1. A majority of the pluripotency genes and the genes known to inhibit various differentiation pathways were also expressed by these cell lines. We also observed that each of these cell lines expressed a unique set of genes that are mutually exclusive from each other. These results represent the first detailed characterization of a set of hESC lines originating from India.

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Introduction

Human embryonic stem cells (hESCs) are derived from the inner cell mass (ICM) of preimplantation embryos. The fundamental characteristics of these cells include pluripotency and the capacity for self-renewal. This confers on hESCs unlimited replicative ability for extended periods of time while maintaining an undifferentiated state along with the capability of differentiating into derivatives of all three

embryonic germ layers (Reubinoff et al., 2000; Odorico et al., 2001). Thus, hESCs are considered to be good candidates for studying various aspects of developmental biology research as well as a source of cells for tissue regeneration and cell replacement therapies (Liew et al., 2005).

Derivation and characterization of hESC lines are important in terms of direct application to human diseases. Since the establishment of the first hESC lines reported by Thomson and his group about a decade ago (Thomson et al., 1998), rapid progress has been made in this research and several studies (Amit and Itskovitz-Eldor, 2002; Cowan et al., 2004; Park et al., 2004; Guhr et al., 2006) have described the derivation and culture of new hESC lines from fresh/frozen morula (Strelchenko et al., 2004) and/or blastocyst stage embryo

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(Mitalipova et al., 2003; Cowan et al., 2004). Despite the use of different protocols for hESC line derivation and culture maintenance, by and large all hESC lines express pluripotency-related genes, e.g., OCT4, NANOG, and SOX2, as well as a battery of cell surface markers such as SSEA3, SSEA4, TRA-1-60, TRA-1-81, CD9, and CD90. Characterizations of 57 hESC lines derived from many different laboratories worldwide by the International Stem Cell Initiative (ISCI) have demonstrated remarkable similarities between the lines, although subtle differences in gene-dependent variations were observed between these cell lines (Adewumi et al., 2007). Comparative studies including large-scale gene expression analysis in multiple hESC lines (Bhattacharya et al., 2004; Richards et al., 2004) are becoming rather common and also more advanced. Despite the apparent overall similarity in the fundamental expression patterns among the hESC lines, important differences have been noted. The differences ranging from the growth rates and differentiation patterns, methylation patterns to karyotypic stability, and changes associated with long-term propagation in culture of hESC lines have been frequently reported (Draper et al., 2004; Hoffman and Carpenter, 2005; Tavakoli et al., 2009). In addition, various hESC lines derived and maintained under identical culture conditions exhibited significant differences in their ability to differentiate toward certain lineages as reported in several cases (Osafune et al., 2008; Mehta et al., in press). These results seem to indicate that different hESC lines may not

possess similar differentiation bias *in vitro* and such differentiation propensity needs to be examined prior to generating an optimal differentiation protocol for mature cell types with potential therapeutic applications.

The great majority of well-characterized hESC lines have been derived from relatively small cross sections of the human population with limited ethnic diversity. In two recent independent reports, investigators have determined the ethnicity of 47 hESC lines using genome-wide single nucleotide polymorphism (SNP) genotyping which reveals that almost all of these lines were derived from subjects of European and Middle Eastern origin (Laurent et al., 2010; Mosher et al., 2010). In light of these findings, it is important that new hESC lines be derived from populations of diverse genetic background so that the differential potential, drug toxicity profiles, and immunological phenotypes (HLA haplotype) of these cell lines can be assessed and compared with the existing hESC lines. In addition, availability of hESC lines from embryo donors of diverse origin and ethnic background may provide a better HLA matching within the population from which the lines are derived. Although the recent development of a generation of differentiated cell types from induced pluripotent stem (iPS) cells may eliminate the possibility of immune rejection after transplantation (Takahashi et al., 2007; Maehr et al., 2009), clinical applicability of such individualized cell therapy remains a distant possibility.

Table 1 Establishment of the Relicell[®]hESC lines

Embryo No.	Stage/grade ^a	Isolation procedure	Embryo attachment	Outgrowth	Timing of separation of overgrowing TE cells after embryo plating	Outcome
1	D6/3AB	Laser	Yes	No	D7	P1
2	D6/3BA	Whole	Yes	No	D7	P1
3	D7/3AA	Laser	Yes	No	D6	P1
4	D6/4BA	Laser	Yes	No	D7	P1
5	D6/4AA	Laser	Yes	Yes	D7	P2
6	D6/3CB	Laser	No	NA	NA	NA
7	D6/3CB	Whole	No	NA	NA	NA
8	D5/4AA	Immunosurgery	Yes	No	D7	P1
9	D5/4AB	Immunosurgery + Mechanical surgery	Yes	Yes	D3	Relicell [®] hES2 cell line
10	D6/5BB	Nat. Hatching	Yes	No	D2	P2
11	D5/4BB	Laser	Yes	No	Not done as ICM was not visible after D2	P0
12	D5/4CB	Whole	Yes	No	D7	P1
13	D5/3BB	Immunosurgery	Yes	No	Not done as ICM was not visible after D2	P0
14	D5/3BB	Immunosurgery	Yes	No	Not done as ICM was not visible after D2	P0
15	D5/4AB	Immunosurgery + Mechanical surgery	Yes	Yes	D4	Relicell [®] hES3 cell line
16	D5/4BC	Immunosurgery + Mechanical surgery	Yes	Yes	D4	Contaminated at P1
17	D6/4CB	Whole	Yes	No	D4	P1
18	D6/4CB	Nat. Hatching	Yes	No	D5	P1

Abbreviations: P, passage; D, day; NA, not applicable.

^aBlastocyst grade was defined according to the criteria presented by Gardner et al. (2000).

^bMechanical removal of TE from the ICM + TE complex on Day 3/4 was done from embryo No. 9 onward.

In order to address some of these issues described above, we (Mandal et al., 2006) along with other groups (Inamdar et al., 2009; Kumar et al., 2009) have reported the generation of hESC lines from the Indian population. In this current study, we describe the successful derivation of two

more new hESC lines from the Indian population: Relicell[®]hES2 and Relicell[®]hES3 from surplus *in vitro* fertilized (IVF) embryos. These new cell lines have been simultaneously maintained in an undifferentiated state in culture for over 1 year. They have been characterized, expanded, frozen, and

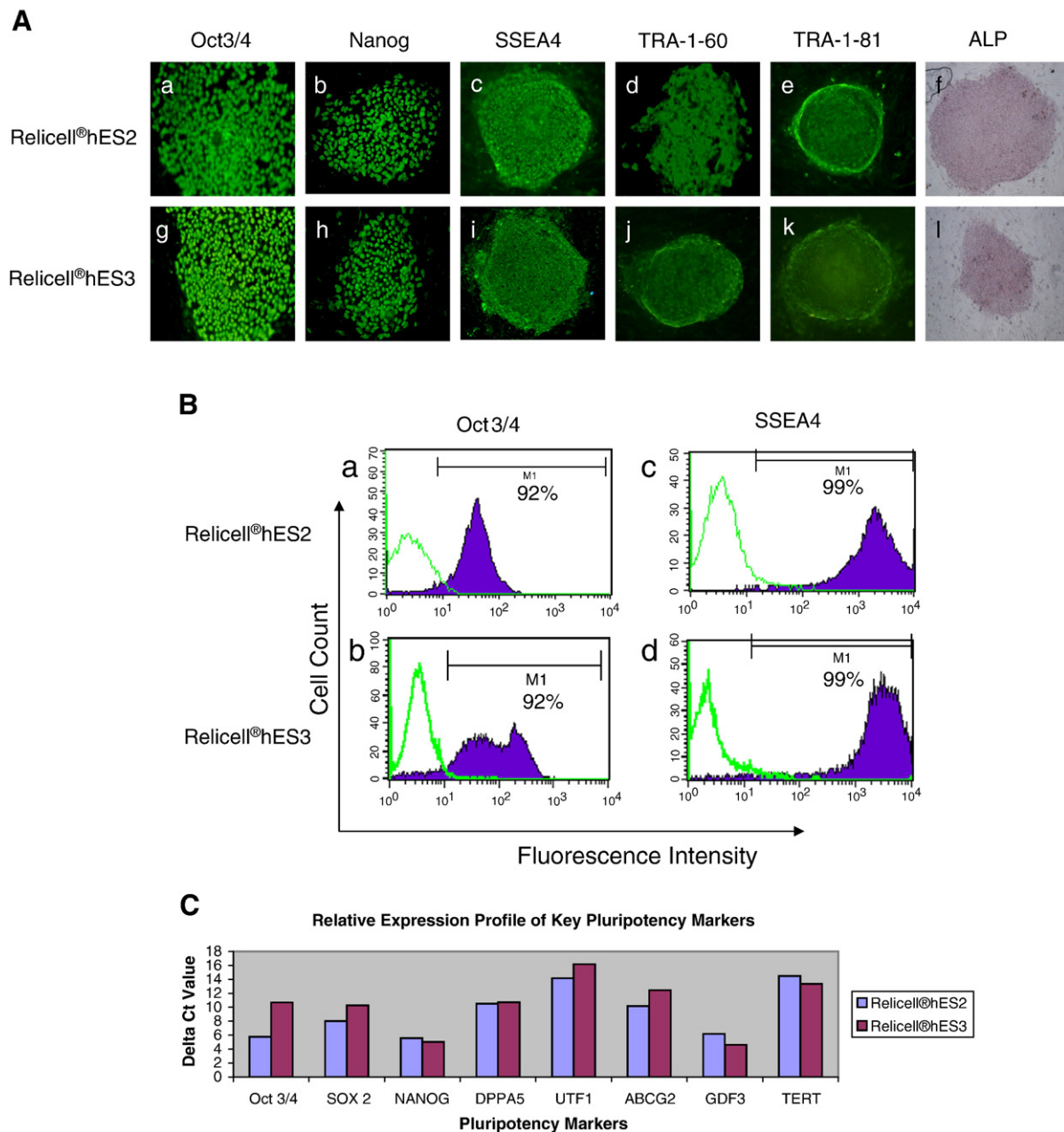


Figure 1 (A) Immunostaining profile of Relicell[®]hESC lines. Undifferentiated cells of each of these cell lines on Day 4 of culture were strongly positive for OCT3/4 (a and g), NANOG (b and h), SSEA-4 (c and i), TRA-1-60 (d and j), TRA-1-81 (e and k), and alkaline phosphatase (f and l). Both primary and secondary controls staining offered negativity. (B) Immunophenotyping of Relicell[®]hESC lines by flow cytometry shows immunoreactivity (shaded histograms) of these cells to OCT3/4 (a and b) and SSEA 4 (c and d). The antibodies were directly labeled with phycoerythrin (PE). Isotype-antibody staining (green line) shows background fluorescence. (C) Molecular profile of Relicell[®]hESC lines. Real-time qRT-PCR analysis shows the expression of the common pluripotent genes OCT3/4, SOX2, NANOG, DPPA5, UTF1, ABCG2, GDF3, and TERT. The expression of each gene was normalized to the corresponding levels of 18 s rRNA gene. Both the cell lines show the presence of these genes with little variations in their expression levels.

thawed with no loss of pluripotency. Furthermore, we have compared the characteristics of these hESC lines in terms of morphology, expression of pluripotency markers, and differentiation capacity both *in vitro* and *in vivo*. Microarray analysis revealed close similarity between our three cell lines, although each one of these lines expresses a unique set of genes that may further contribute to our understanding of human ES cell biology.

Results

Derivation and culture of hESC lines

Of the 18 blastocysts, immunosurgery and laser ablation were performed on 6 samples each, whole embryo manipulation was done on 4, and natural hatching was done for 2 (Table 1). The new hESC lines Relicell[®]hES2 and Relicell[®]hES3 were derived with a combinatorial approach of immunosurgery and mechanical isolation of ICM cells. The blastocysts (Supplementary Fig. 1, a and d) were initially exposed to antibody and complement-mediated lysis for trophoblast (TE) removal. However, with this exposure followed by repeated but gentle pipetting of the blastocyst, the TE cells did not lyse completely and most of these cells were clumped together with the ICM. Without risking further damage to the embryos, the mass was plated on a mitotically inactivated MEF feeder layer. In our earlier experience, we have often observed that the overgrowth of TE cells prevented ES cell proliferation. Therefore, in this case, the TE cells were mechanically separated during the third or fourth day of culture. This allowed the ICM to proliferate and subsequently transform into an ES-like colony (Supplementary Fig. 1, b and e). The initial colony was then mechanically passaged approximately a week later.

The new hESC lines formed distinct colonies with the characteristic morphology of human ES cells, such as densely packed cells with high nuclear to cytoplasmic ratios and having clearly defined borders (Supplementary Fig. 1, c and f). The cell lines have been in culture for over 1 year and have been propagated for more than 80 passages so far.

Marker expression in the hESC lines

The undifferentiated state of the hESC lines was confirmed by evaluating several cell surface markers by immunofluorescence analysis. Colonies of Relicell[®]hES2 (p10) and Relicell[®]hES3 (p11) cell lines uniformly expressed markers such as OCT-3/4, NANOG, SSEA4, TRA-1-60, and TRA-1-81 (Fig. 1A, a–e and g–k). The cells from each of these cell lines were highly positive for Oct 3/4 (92%) and SSEA4 (99%) as quantitated by flow cytometric analysis (Fig. 1B). As expected the hESC colonies displayed strong alkaline phosphatase activity (Fig. 1A, f and l).

Real-time RT-PCR was used to quantify the levels of mRNA expression of 8 selected genes (OCT3/4, SOX2, NANOG, DPPA5, UTF1, ABCG2, GDF3, and TERT) to determine the undifferentiated ESC state. All the genes were highly expressed in Relicell[®]hES2 and Relicell[®]hES3 cell lines (Fig. 1C).

Microarray analysis of the hESC lines

Gene expression profiles of Relicell[®]hESC lines

In order to determine the similarities and differences between the two new Relicell[®]hESC lines, we performed a whole genome array analysis using Illumina BeadChip with arrays containing 24 000 transcripts derived from the Human RefSeq database which included both full-length and spliced variants. Gene expression analysis of Relicell[®]hES1 by microarray has been reported previously (Pal et al., 2007). The rationale for including Relicell[®]hES1 in this study was to perform parallel genome-wide expression analysis of all the three Indian cell lines that were derived and established in our laboratory.

Although the three Indian hESC lines were closely related to each other in comparison to a well-studied line, BG01 (Zeng et al., 2004; Bhattacharya et al., 2004), gene expression profiles between Relicell[®]hES2 and Relicell[®]hES3 were more similar to each other than with Relicell[®]hES1 (Fig. 2A). Our fourth hESC line, Relicell[®]hES4 derived from a PGD diagnosed embryo, was clustered together with Relicell[®]hES1 as determined by the Genome Studio cluster package. The complete characterization and differentiation profiles of Relicell[®]hES4 will be published elsewhere. The R^2 values between each

Figure 2 (A) Dendrogram showing hierarchical clustering of total genes expressed in all Relicell[®]hESC lines as well as in BG01 and MEF cells. Clustering was carried out using the cluster analysis package of Genome Studio software. The Euclidean metric algorithm was used for the dendrogram formation. The clustering shows close association between the four Relicell[®]hESC lines compared to BG01. * Relicell[®]hES4 cell line was derived from a PGD analyzed embryo. The detailed report on characterization of this cell line will be published elsewhere. (B) Differences in relative expression levels of undifferentiated cell markers among the three undifferentiated Relicell[®]hESC lines. The expression levels of 54 undifferentiated marker genes were compared to GAPDH and the expression levels were found comparable in most of them. CD24, DPPA4, and LIN28 were found to be expressed at very high levels in all the cell lines. The high expression of these 54 genes imply that all three Relicell[®]hESC lines are pluripotent. The data show the consistency of the expression levels of the undifferentiated marker genes (OCT3/4, NANOG, and SOX2) compared with the results of the qRT-PCR analysis (Fig. 1B). (C) A heat map representation of all the 79 genes from the list of 85 undifferentiated marker genes (6 genes did not show any detectable probe on the Microarray chip and are therefore excluded from this list). Each row represents the relative levels of expression of a single marker gene. Samples 1–3 included three groups of cells, Relicell[®]hES1, Relicell[®]hES2, and Relicell[®]hES3, respectively. High expressions relative to mean are colored red. Low expressions are colored green. Black represents no significant change in expression levels between mean and sample. (D) Differences in relative expression levels of differentiated lineage-specific cell markers among the three undifferentiated Relicell[®]hESC lines. Expression levels of 22 representative differentiated lineage-specific markers expressed in all the three Relicell[®]hESC lines have been shown in comparison to GAPDH. All markers have been obtained within the $P < 0.01$ cutoff range. Only LAMC1 and TUBB showed some correlation to GAPDH expression whereas other markers were found to be weakly expressed. DCN, GSC, LAMB1, MAP2, and T (Brachury) are seen to be differentially expressed.

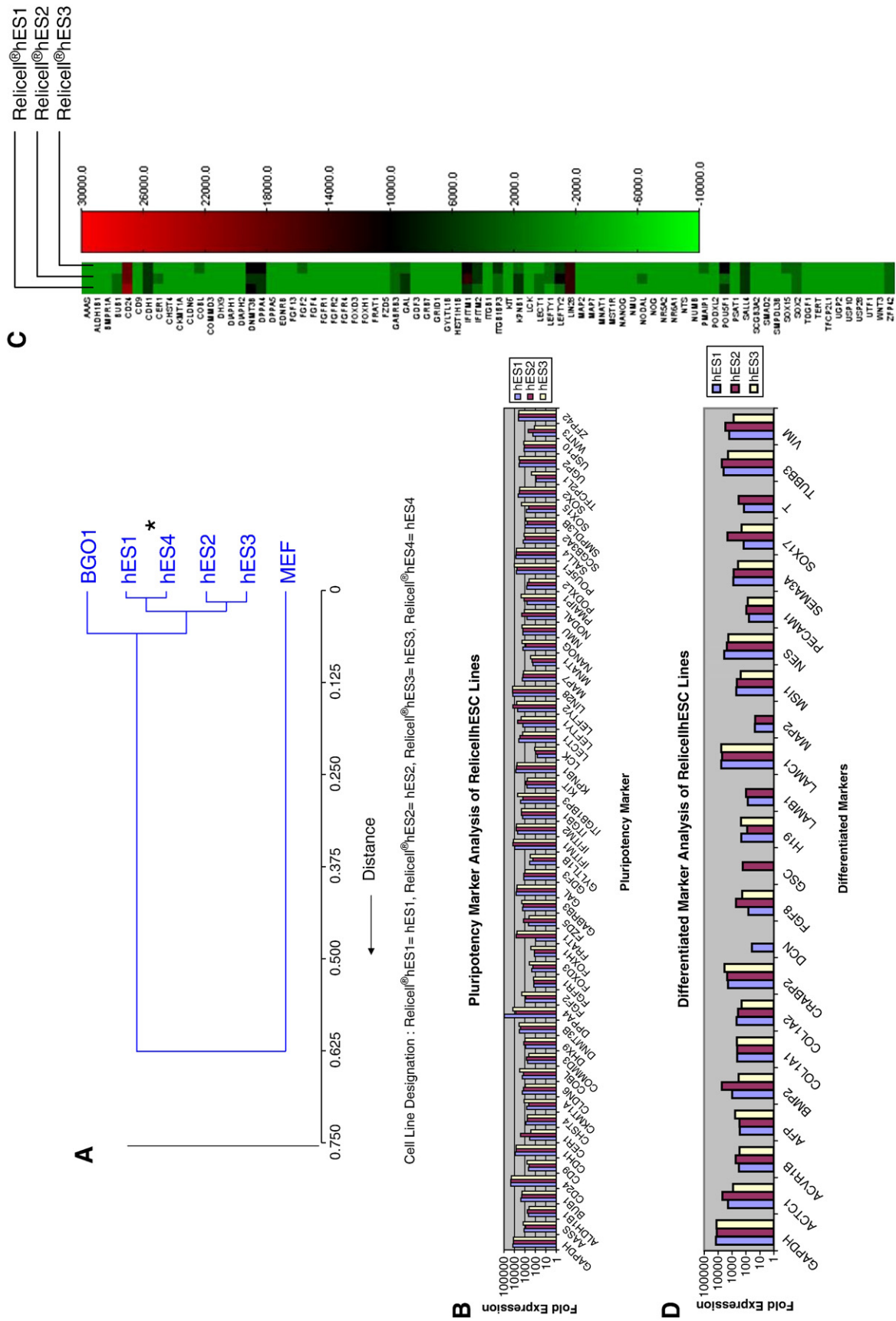


Table 2 Differential expression of thirteen undifferentiated markers in Relicell[®]hESC lines

Gene symbol	Accession	Chromosome location	Definition	Gene expression		
				hES1	hES2	hES3
BMPR1A	NM_004329.2	10	<i>Homo sapiens</i> bone morphogenetic protein receptor, type IA (BMPR1A), mRNA.	^a ND	+	+
DPPA5	NM_001025290.1	6	<i>Homo sapiens</i> developmental pluripotency-associated 5 (DPPA5), mRNA.	+	ND	ND
DIAPH1	NM_005219.2	5	<i>Homo sapiens</i> diaphanous homolog 1 (<i>Drosophila</i>) (DIAPH1), mRNA.	+	ND	+
FGF13	NM_033642.1	X	<i>Homo sapiens</i> fibroblast growth factor 13 (FGF13), transcript variant 1B, mRNA.	ND	+	ND
GRB7	NM_005310.2	17	<i>Homo sapiens</i> growth factor receptor-bound protein 7 (GRB7), transcript variant 1, mRNA.	ND	+	+
MAP2	NM_002374.3	2	<i>Homo sapiens</i> microtubule-associated protein 2 (MAP2), transcript variant 1, mRNA.	+	+	ND
NOG	NM_005450.2	17	<i>Homo sapiens</i> noggin (NOG), mRNA.	ND	+	ND
NR5A2	NM_205860.1	1	<i>Homo sapiens</i> nuclear receptor subfamily 5, group A, member 2 (NR5A2), transcript variant 1, mRNA.	+	ND	+
NR6A1	NM_001489.3	9	<i>Homo sapiens</i> nuclear receptor subfamily 6, group A, member 1 (NR6A1), transcript variant 2, mRNA.	ND	+	ND
NUMB	NM_003744.5	14	<i>Homo sapiens</i> numb homolog (<i>Drosophila</i>) (NUMB), transcript variant 3, mRNA.	ND	+	+
TDGF1	NM_003212.1	3	<i>Homo sapiens</i> teratocarcinoma-derived growth factor 1 (TDGF1), mRNA.	+	ND	ND
TERT	NM_198253.2	5	<i>Homo sapiens</i> telomerase reverse transcriptase (TERT), transcript variant 1, mRNA.	+	ND	+
UTF1	NM_003577.2	10	<i>Homo sapiens</i> undifferentiated embryonic cell transcription factor 1 (UTF1), mRNA.	+	ND	+

Abbreviation: ND – not detected.

duplicate sample were found to be around 0.99, confirming the reproducibility of the assay. On filtering the total number of genes detected by the array (18391 for each cell line) based on $P < 0.01$ criteria, we found that the number of genes significantly expressed in Relicell[®]hES1, Relicell[®]hES2, and Relicell[®]hES3 were 9245, 9253, and 9276, respectively, out of which the number of genes common to all three lines was 7236. The remaining genes consisting of 2009 for Relicell[®]hES1, 2017 for Relicell[®]hES2, and 2040 for Relicell[®]hES3 were differentially expressed by these cell lines.

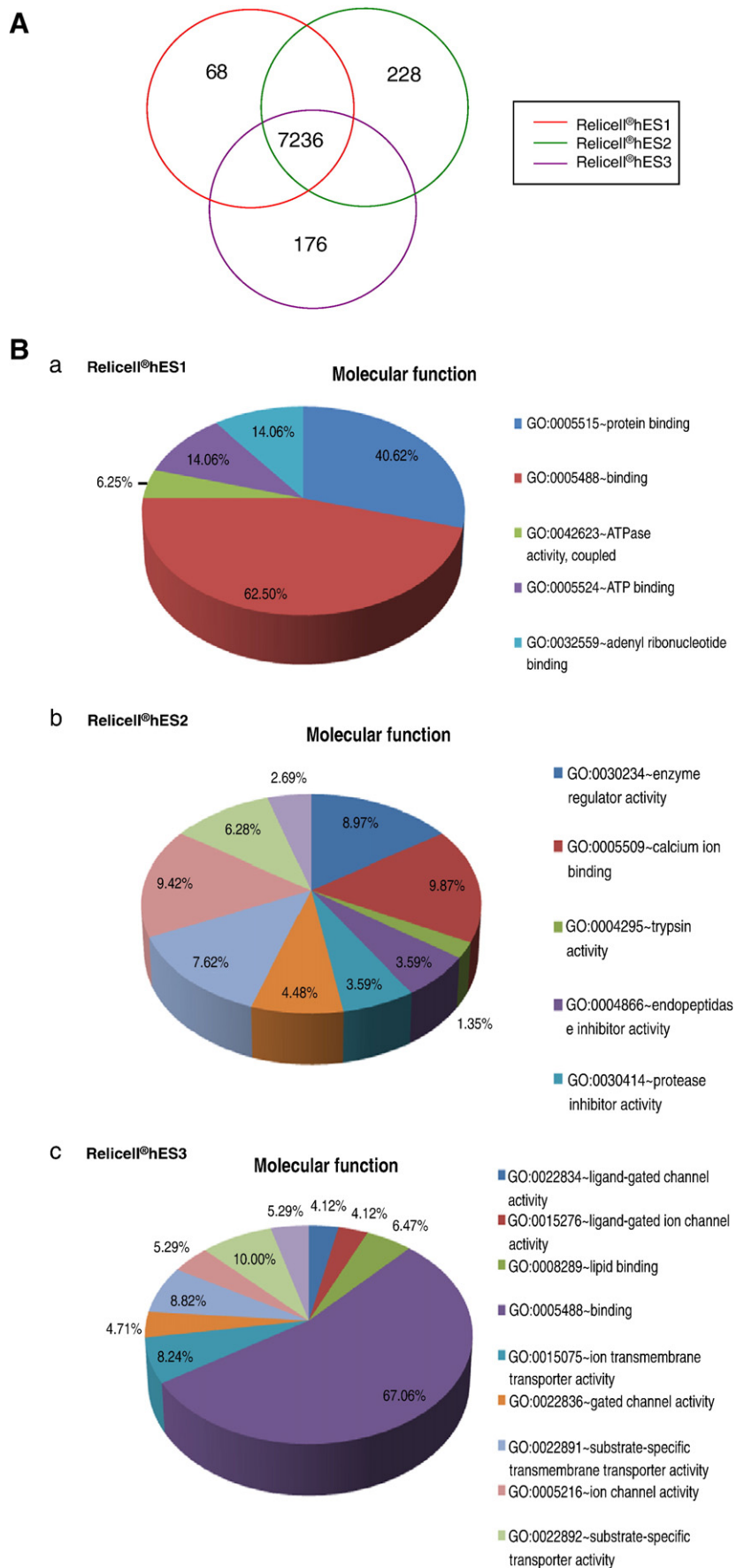
As the Relicell[®]hESCs were cultured over MEF feeders, we probed the possibility of MEF contamination with the hESC samples. In a separate set of experiments, we spotted MEF cRNA along with the hESC samples and found that only 1200 genes were significantly expressed ($P < 0.01$) (data not shown) as compared to 9000 plus genes in hESC lines. However, except for IFITM1, LAMC1, ACTC1, CGB5, and ISL1, none of the pluripotency-related genes were seen to

coexpress in the MEF feeder sample. The coexpression of the above-noted genes could be due to cross-reacting probes present in Illumina BeadChip.

Expression of genes known to regulate pluripotency in Relicell[®]hESC lines

In recent years, a large dataset on gene expression of undifferentiated hESCs has been generated using large-scale experiments such as MPSS, SAGE, and microarray (Richards et al., 2004; Brandenberger et al., 2004; Liu et al., 2006). The common goal of all such extensive studies was to come up with some characteristic markers significant for determining the pluripotent state of hESCs. In reference to all these published data on hESCs, we came up with a list of 85 genes which have thus far been reported to be enriched in hESCs by different investigators worldwide (Abeyta et al., 2004; Skottman et al., 2005; Liu et al., 2006). This list of 85 genes is the first cumulative list of genes established as

Figure 3 (A) Venn diagram illustrating the digital gene expression profile of the Relicell[®]hESC lines and the intersection of genes between the cell lines. All the cell lines were grown under the same culture conditions. Only genes with $P < 0.01$ were taken for this analysis. It shows that around 78% (7236 out of 9308) of the genes are common to all the three lines whereas only 0.7, 2.4, and 1.9% of genes are uniquely expressed by Relicell[®]hES1, Relicell[®]hES2, and Relicell[®]hES3, respectively. The large overlap in genes (7236) commonly expressed among the three lines suggests the presence of a stable core of stemness transcriptome. (B) Pie chart representing molecular function of unique signature genes in the Relicell[®]hESC lines. This chart shows the expression of different molecular function-related genes in all three individual lines. The functional clustering was done by the DAVID gene ontology tool (GoEASY). The clustering shows the expression of several types of binding genes in the three lines identifying their similarity amidst uniqueness. Some of these genes have been assigned to more than one molecular function.



representative markers of hESC pluripotency. The list also included the core set of 20 genes established as pluripotency markers by the ISCI studies on 57 hESC lines (Adewumi et al., 2007). Fifty-four of these 85 genes were found to be expressed in all three Relicell[®]hESC lines (Fig. 2B). Thirteen genes were found to be variably expressed among the lines (Table 2) while 12 genes did not show any significant expression (Supplementary Table 1) in these three lines. Six genes from this list, namely EBAF, BRIX, XIST, LIN41, ZNF206, and TDGF1P, were not detected in the array. Besides the well-established pluripotency genes such as OCT3/4, NANOG, SOX2, REX, GDF3, LEFTB, LIN28, and DNMT3B, many other genes such as CD24, DPPA4, NODAL, and FOXD3 are believed to be important in maintaining the undifferentiated state. Most of these markers were enriched in all three Relicell[®]hESC lines. Twelve of the 20 ISCI study genes were present in these three lines (Supplementary Figs. 2A and C) whereas 4 genes, TERT, UTF1, GRB7, and TDGF1, were variably expressed. We could not detect the expression of the remaining 4 genes (FGF4, BRIX, XIST, and EBAF) reported in the ISCI study in any of our cell lines. These data taken together suggest that all three Relicell[®]hESC lines show similar, but not identical, patterns of gene expression with respect to the markers that are indicative of pluripotency. The expression levels of the pluripotency markers clearly indicate the self-renewal state of our hESC lines. A complete analysis of all the 79 pluripotency markers has been depicted in the form of a heat map (Fig. 2C).

Next, we determined the expression pattern of few differentiated cell markers that were shown to be present in other well-characterized hESC lines at the undifferentiated stage. Of the 13 genes that were examined in the ISCI report (Adewumi et al., 2007) ACTC1, AFP, COL1A1, COL1A2, EOMES, LAMA1, and Sox17 were found to be expressed by all three Relicell[®]hESC lines (Supplementary Figs. 2B and 2C). Interestingly, GATA4 was detected only in Relicell[®]hES2 and Relicell[®]hES3 cell lines but not in Relicell[®]hES1. In contrast, GATA6 was expressed only by Relicell[®]hES2. The remaining 4 genes were not expressed by any of our lines. We also

measured the expression of 48 genes that has been shown to be associated with the three germ layers and observed that 17 of these genes are expressed by all three lines and 5 genes (DCN, GSC, LAMB1, MAP2, and T) show a variable expression among the lines (Fig. 2D). It is not clear whether these differentiated cell-associated genes are expressed by the undifferentiated hESCs or in a population of cells that have spontaneously differentiated in our culture. However, none of the differentiated markers such as ALB, NEUROD1, INS, MHC, OLIG2, and CYP3A4 known to be expressed in mature cells were present in these cell lines, indicating their undifferentiated state.

Presence of unique signature genes in Relicell[®]hESC lines

A Venn diagram depiction of the unique and common genes is shown in Fig. 3A. While 68 unique genes were found in Relicell[®]hES1, the number went up to 228 in Relicell[®]hES2 and 176 in Relicell[®]hES3. Further elucidation of the nature of the unique signature genes classified them into three ontology domains that included biological processes, cellular components, and molecular function. It was observed that a high percentage of the preferred ontology groups included protein binding genes (PRKCZ, ILK, GABBR1, DCN, etc. in Relicell[®]hES1, Fig. 3B, a), calcium ion binding genes (STAT4, MYL4, MIER3, MAN1A2, etc. in Relicell[®]hES2, Fig. 3B, b), lipid binding genes (ULBP1, DGKA, APOA4, SNAP91, etc. in Relicell[®]hES3, Fig. 3B, c). Other closely related ontology types such as ATP binding, transcription factors, transcription regulation, voltage gated ion channel activity, and zinc ion binding were also found to functionally designate the unique genes. The significance of these markers in the context of pluripotency or differentiation ability of these hESC lines is difficult to ascertain at this time.

The list of unique genes also included genes with no known functions (Table 3). The number of unique genes with no known function was found to be 1 for Relicell[®]hES1, 3 for Relicell[®]hES2, and 4 for Relicell[®]hES3. Four of these 8 genes present in Relicell[®]hES1, Relicell[®]hES2, and Relicell[®]hES3 with unknown function were mapped in chromosome 7. The

Table 3 List of unique genes with no known function

Cell line	Gene symbol	Illumina search key	Chromosome location	Definition
Relicell [®] hES1	LOC255374	ILMN_5827	7	<i>Homo sapiens</i> similar to hypothetical protein MGC49416 (LOC255374), mRNA.
Relicell [®] hES2	FLJ10324	ILMN_3564	7	<i>Homo sapiens</i> hypothetical protein FLJ10324 (FLJ10324), mRNA.
	FLJ13236	ILMN_28329	12	<i>Homo sapiens</i> hypothetical protein FLJ13236 (FLJ13236), mRNA.
	LOC51057	ILMN_30040	2	<i>Homo sapiens</i> hypothetical protein LOC51057 (LOC51057), transcript variant 1, mRNA.
Relicell [®] hES3	FLJ22374	ILMN_22269	7	<i>Homo sapiens</i> hypothetical protein FLJ22374 (FLJ22374), mRNA.
	LOC222967	ILMN_175454	7	<i>Homo sapiens</i> hypothetical protein LOC222967 (LOC222967), mRNA.
	LOC51149	ILMN_15092	5	<i>Homo sapiens</i> hypothetical LOC51149 (LOC51149), transcript variant 2, mRNA.
	LOC606495	ILMN_4186	1	<i>Homo sapiens</i> hypothetical protein LOC606495 (LOC606495), mRNA.

functional determination of these unknown genes may reveal important clues to the signature identities of the three Relicell[®]hESC lines.

Signaling pathway analysis

Several different signaling pathways have been reported to be involved in maintaining hESC pluripotency. Here we have looked into the expression patterns of the participating components (www.genome.jp/kegg/pathway.html) of WNT, TGF β , and Nodal/Activin pathways in all the Relicell[®]hESC lines. The WNT target genes c-MYC, JUN, FOS, and CycD were found to be present in all the three cell lines. Though there is a variable expression of FRZ receptors and β -catenin is not detected in any of the lines, nevertheless a β -catenin-like protein CTNNB1 is found to be considerably enriched in all the cell lines. The TGF- β pathway genes such as TGFBR2, TGFBR3, SMAD3, and SMAD4 are present in all the lines. The inhibitors such as THBS, CUL1, and RBX were found to be enriched in all the lines. The TGF- β target genes EP300, SP1, and E2F4/E2F5 are also present in these lines. We also observed very high expression of LEFTY and FST in the hESC lines, indicating possible inhibition of the Nodal/Activin pathway, though Nodal is found to be expressed in all our lines. A tabular depiction of the genes from different pathways and their presence in the three hESC lines is shown in Supplementary Table 2.

Genetic stability and identity of the hESC lines

Karyotype analysis showed that the two new hESC lines maintained a stable diploid normal karyotype. Both Relicell[®]hES2 and Relicell[®]hES3 showed normal 46XX distribution (Supplementary Fig. 3). Frozen–thawed samples of these cell lines showed normal karyotype as well.

We have developed a comprehensive database of DNA profiles for each of our cell line based on HLA typing and STR loci distribution. Our results from the two new hESC lines revealed that these cells are of heterozygous HLA genotype except for homozygous DQB locus in Relicell[®]hES3 cell line (Supplementary Table 3). The data also showed that Relicell[®]hES2 and Relicell[®]hES3 cell lines have different HLA genotypes which may be useful for identification. Sixteen STR loci were analyzed for the hESC lines and each cell line showed distinct sets of STR loci, indicating that they were derived from genetically different embryos (Supplementary Table 4).

In vitro and *In vivo* differentiation potential

Spontaneous differentiation of hESCs into various lineages via embryoid body (EB) formation was determined by marker expression at the molecular level. qRT-PCR results show that both hESC lines expressed genes of ectoderm (Nestin, Beta-tubulin, and NFH; Fig. 4A, panel a), mesoderm (HAND1, T, and GATA4; Fig. 4A, panel b), and endoderm (AFP, Sox17, and HNF4a; Fig. 4A, panel c) lineages. The level of expression of a particular gene was found to vary at different stages of differentiation for each cell line, indicating differential up or down regulation during the differentiation period. Since the Relicell[®]hES2 cell line had not generated teratoma in SCID mice so far (see below), these cells were allowed to

spontaneously differentiate into EBs to determine the presence of cellular markers belonging to the three germinal lineages. Immunostaining of EBs showed that the differentiated cells of Relicell[®]hES2 expressed ectodermal marker, Nestin (Fig. 4B, panel a), mesodermal marker, GATA4 (Fig. 4B, panel b), and endodermal marker, HNF4a (Fig. 4B, panel c).

The *in vivo* differentiation potential of the two lines was further evaluated by observing teratoma formation in SCID mice. The developing or fully developed tumors were dissected between 8 and 12 weeks after the injection of hESCs from the Relicell[®]hES3 line. The resulting teratomas contained derivatives of all the three germ layers: ectoderm (neurons, keratin, and hair follicles), mesoderm (bone, cartilage, artery, and adipocytes), and endoderm (respiratory track epithelium, intestinal epithelium, and renal development) (Fig. 4C). This demonstrates that this hESC line is pluripotent and capable of differentiating into all three germ layers *in vivo*. No teratoma formation has yet been observed with the Relicell[®]hES2 cell line in immunocompromised mice.

Discussion

Human embryonic stem cells are commonly isolated from the ICM of the expanded blastocyst or compacted morula, obtained from spare donated embryos after IVF treatment. In this study, two new hESC lines were derived from a similar source of embryos obtained from the Indian population. The method used for derivation was chosen keeping in view the embryo quality and specifically the size of the ICM. For good quality blastocysts, immunosurgery or laser ablation was used whereas for average and poor quality as well as for hatching blastocyst, a whole embryo plating method was performed (Kim et al., 2005). We observed that the mechanical separation of the ICM from the TE cells during the first few days of the culture was useful in terms of ICM outgrowth and establishment of ES cell colony. This is applicable both for whole embryo culture and cases where TE cells remain attached to ICM post immunosurgery. Although other studies (Heins et al., 2004; Ellerstrom et al., 2006) have used similar techniques to isolate the ICM for derivation of hESC lines, none of them suggested such early separation of TE cells followed by fresh plating of ICM. Thus it appears from our data that mechanical teasing of hyperproliferative TE cells is a better method for increasing the chances of ICM survival and ES cell line derivation.

In accordance with previously reported studies, we have observed the usual characteristics of hESCs in these newly established lines in terms of cell marker expression pattern, stability, identity, and ability to differentiate both *in vitro* and *in vivo*. All these cell lines expressed the stem cell-specific surface markers such as SSEA4, TRA-1-60, TRA-1-81, and transcription factors OCT3/4, NANOG, and SOX2, and maintained their genomic stability and pluripotency for more than 80 passages, as demonstrated by karyotyping and the ability to spontaneously differentiate under *in vitro* and *in vivo* conditions. HLA profile and STR genotyping status have established the unique identity for each of these hESC lines. Relicell[®]hES2 and Relicell[®]hES3 cells readily form EBs which

appeared very similar morphologically, but we observed some differences in the expression of lineage-specific genes. Representation of the three germ layers was observed in the Relicell[®]hESC cell line in teratoma which further elucidated its pluripotent nature.

To our knowledge, this is the first comprehensive study of genome-wide microarray analysis of three hESC lines of Indian origin. Of the approximate 9000 genes expressed by the Relicell[®]hESC lines, 7236 transcripts are common to all three cell lines including the conventional pluripotency markers such as OCT3/4, NANOG, SOX2, DNMT3B, LIN28, and LEFTY 1/2. The presence of OCT3/4, SOX2, and NANOG was further confirmed by qRT-PCR as well as by immunostaining. When we compared our data with those of the ISCI report, we found that all but 4 genes are present in our lines. Though TERT, UTF, and TDGF were not detected universally in our lines by Illumina Bead array, their expression was confirmed by RT/qRT-PCR, indicating one of the potential shortcomings of microarray analysis. Our analysis of 85 pluripotency-related genes revealed that 54 of these genes were common among all the three lines while 13 genes were differentially expressed. The variable expression of DPPA5, TERT, TDGF1, and UTF1 among various Relicell[®]hESC lines shows an underlying difference between the three characteristically similar lines. Eighteen of the 85 genes absent in our three lines included 6 genes that had no detectable probes in the chip. This group of genes includes LIN41, EBAF, XIST, ZNF206, TDGF1P, and BRIX. Similar cases of undetectable probes for markers have been reported previously (Liu et al., 2006).

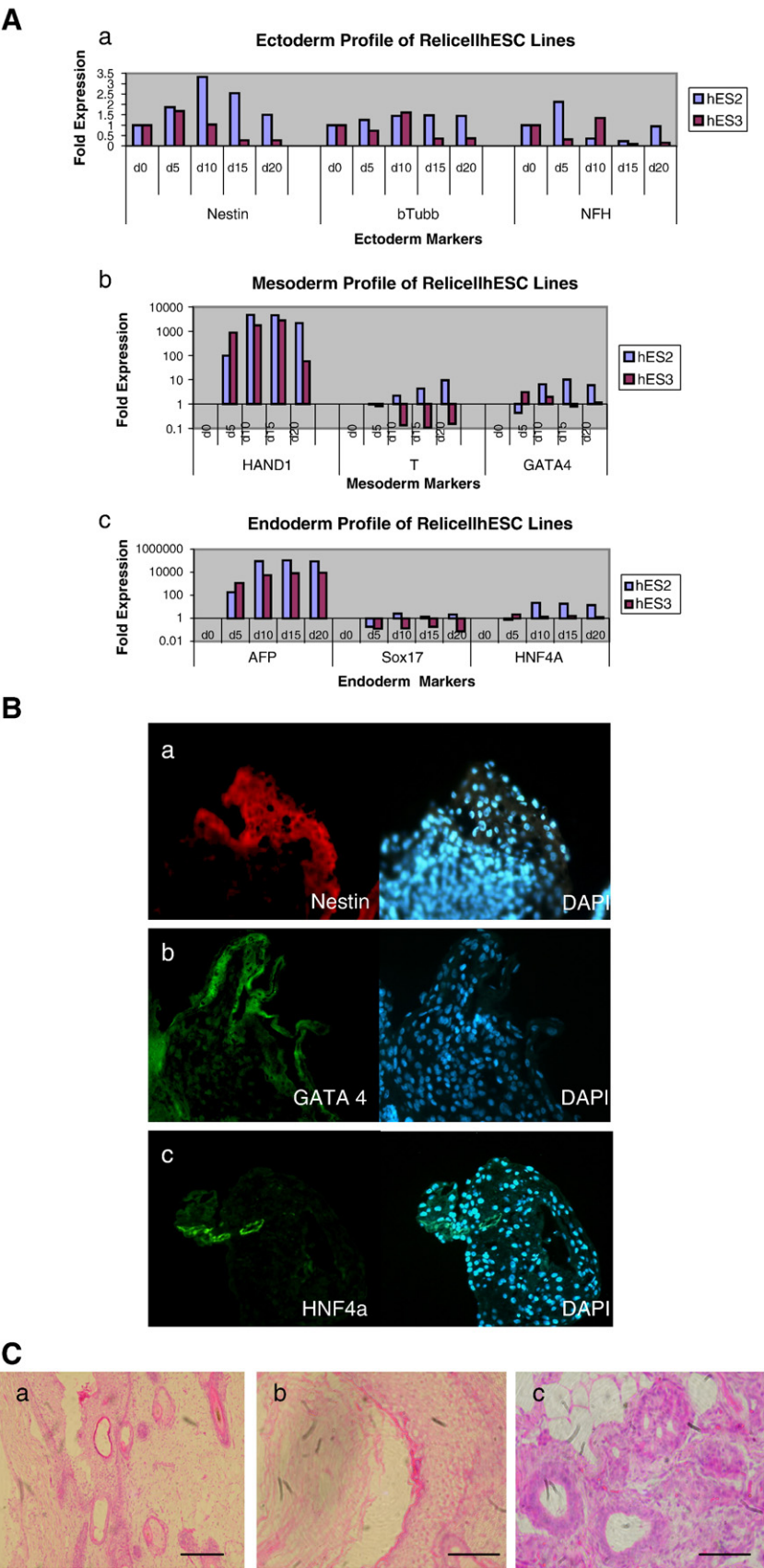
In addition to the commonly expressed and unique signature genes, all three Relicell[®]hESC lines expressed certain differentiation-associated markers such as SOX17, GSC, DCN, GATA4, and GATA6. Brachury (T) expression was detected in undifferentiated Relicell[®]hES1 and Relicell[®]hES2 cells, but not in Relicell[®]hES3. Similarly, GSC expression was observed in Relicell[®]hES2 only and DCN gene in the Relicell[®]hES1 cell line. Expression of differentiated cell-specific markers by undifferentiated hESCs has been reported earlier (Adewumi et al., 2007; Zeng et al., 2004). Considering the fact that hyperproliferative hESCs are in a dynamic state, spontaneous differentiation toward trophectoderm and other extraembryonic lineage of cells may account for the differentiated cell marker expression. In addition, the expression of these genes may be indicative of

differentiation bias of these cell lines toward a specific lineage. This remains a hypothesis that needs to be experimentally authenticated.

Signaling pathway analysis also revealed important clues to the molecular state of the hESCs. Components of crucial pathways reported to be involved in maintaining pluripotency such as WNT and TGF β were found to be commonly present in all three lines. WNT signaling has been implicated in the self-renewal of hESCs (Sato et al., 2004). Several WNT signaling genes such as WNT3, CTNNBLP1, LEF, TCF3, and CycD are commonly present in all three Relicell[®]hESC lines. Expression of TCF3 has been reported to be involved in maintenance of pluripotency in mESCs (Tam et al., 2008). WNT3a is variably present between these lines. Though down regulation of GSK3 β is involved in β -catenin stabilization, low levels of the inhibitor were found to be present in all the lines. Microarray data presented here show that many of the TGF β signaling components are present in all three lines. TGF β pathways are likely to be critical for the maintenance of the undifferentiated state of hESCs (Sato et al., 2003; Brandenberger et al., 2004). The Activin/Nodal pathway genes such as ACVR1 and SMAD2/4 are present in all these lines. Potent inhibitors of the activin/nodal pathway such as LEFTY2 and FST are found to be considerably enriched, indicating a tight regulation of the pathways in our cell lines. Similarly, for the BMP pathway the product ID1 is enriched in all the three Relicell[®]hESC lines (data not shown), suggesting that BMP signaling is blocked in these cell to prevent differentiation or to maintain the undifferentiated state. ERK (MAPK1) which is an inhibitor of this pathway was also found to be present in all the three lines. An overall analysis of the signaling pathways indicates an intriguing molecular environment within the hESC lines wherein both antagonists and agonists of the participating pathways are present. The coordinated expression of all these molecules indicates that these signaling pathways are important and are tightly controlled for proper growth and differentiation of hESCs and in maintaining the undifferentiated state of these cells.

In addition to the hESC lines reported here, other groups have recently reported derivation of new hESC lines from the Indian population (Inamdar et al., 2009; Kumar et al., 2009). In light of the enormous genetic diversity that exists within the Indian population, comparative data on genetic and ethnic background of these hESC lines may provide important

Figure 4 (A) *In vitro* differentiation potential of Relicell[®]hESC lines. qRT-PCR analysis shows differences in the expression levels of ectoderm-, mesoderm-, and endoderm-specific genes between the two Relicell[®]hESC lines. The Y-axis plots the fold expression compared to its own undifferentiated cells. Panel (a) shows a comparison of expression levels of the ectodermal-specific genes at Days 5, 10, 15, and 20 of differentiation. Relicell[®]hES2 exhibited a higher expression level of Nestin, NFH, and Tubulin as compared to Relicell[®]hES3. Panel (b) shows a comparison of expression levels of the mesoderm-specific genes at Days 5, 10, 15, and 20 of differentiation. Relicell[®]hES2 and Relicell[®]hES3 showed high expression levels of HAND1 whereas only Relicell[®]hES2 showed expression of GATA4 and T till Day 20. Panel (c) shows a comparison of expression levels of the endodermal-specific genes at Days 5, 10, 15, and 20 of differentiation. Relicell[®]hES2 and Relicell[®]hES3 exhibited a higher expression level of AFP while Relicell[®]hES2 alone showed expression levels of SOX17 and HNF4a till Day 20 of differentiation. (B) Immunofluorescence staining of spontaneously differentiated Day 30 embryoid body sections of the Relicell[®]hES2 cell line shows high expression for ectodermal lineage marker, nestin (a), mesodermal marker, GATA4 (b), and endodermal lineage marker, HNF4a (c), along with respective nuclear staining with DAPI. Scale bar = 200 μ m (a and b) and 100 μ m (c). (C) *In vivo* differentiation potential as shown by histological analysis of teratomas developed by Relicell[®]hES3 cells in SCID female mice. Sections were examined histologically after Hematoxylin and Eosin staining. Teratomas contained multidifferentiated tissues. Shown here are the derivatives of ectoderm, surface epithelium along with sebaceous gland and hair follicles (a); mesoderm, artery (b); and endoderm, renal development (c). Scale bar = 100 μ m.



information regarding the usability of these cells for drug screening and regenerative medicine applications.

Conclusion

We have established two new hESC lines and characterized them extensively. At present, our lab has developed the largest number of characterized hESC lines in India. Determination of the differentiation propensity of these cell lines will help us to understand the finer differences among these lines in future experiments. These studies are currently under investigation.

Materials and methods

Derivation of human ES cell lines

For derivation of hESC lines, spare embryos generated at our IVF center were used. These were donated by the patients after their informed consent and Institutional Committee for Stem Cell Research and Therapy (IC-SCRT) approval was obtained for this research. Eighteen such embryos were cultured to the blastocyst stage as described previously (Mandal et al., 2006). The blastocysts were graded according to the Gardner et al. method (2000). To isolate ES cells, mainly four techniques were applied. (1) Immunosurgery was done as follows; the zona pellucida (ZP) of the blastocysts was removed by Pronase (Sigma, 0.5%, 90 s) digestion. The zona-free blastocysts were incubated with anti-human whole serum antibody (Sigma, 1:10, 30 min.) and then exposed to guinea pig complement (Sigma, 1:4, 15 min.). The embryo mass was then plated on an in-house-derived mouse embryonic fibroblast (MEF) feeder layer which was mitotically inactivated using mitomycin C (Sigma, 10 µg/ml). The media were partially exchanged 2 days later. On the third day of primary plating, the ICM clump was removed mechanically from the rest of the trophectoderm monolayer with a finely pulled Pasteur pipette and replated on a fresh feeder layer. (2) Laser ablation was achieved using a continuous 1.48 µm noncontact diode laser (Saturn Laser System, Research Instruments Limited, UK) for thermal dissolution of the ZP. This was followed by TE cell ablation, by giving 3–4 pulses to cause photolysis. The resulting embryo mass was then washed several times in ES medium and placed on a feeder layer. (3) For poor or average quality blastocysts, the blastocysts were treated with Pronase (Sigma 0.5%, 90 s) followed by direct plating on a feeder layer dish. (4) For deriving hESC lines from Day 6 spontaneously hatching blastocysts, the blastocysts were directly plated on the feeder layer to allow natural hatching to take place. After the embryo was observed to have attached on the feeder plate, mechanical removal of ICM was performed 3 days later to derive the ES cells as described above.

Every 5 days, colonies were subcultured by mechanical dissociation as described earlier (Mandal et al., 2006). Culture media were changed every day. The hESCs were propagated in medium containing 79% DMEM/F12 (Invitrogen), 20% ES tested FBS (Hyclone, USA), 1% nonessential amino acid solution (Invitrogen), 1 mM glutamine (Invitrogen), 0.1% β-mercaptoethanol (Sigma), 15 ng/ml basic

fibroblast growth factor (bFGF; R&D Systems), and 10 ng/ml human leukemia inhibitory factor (hLIF, Sigma). FBS was replaced with 20% of serum replacement (Invitrogen) after the first 15 passages. Also hLIF was withdrawn from the media during this time.

Immunofluorescence, alkaline phosphatase staining, and flow cytometry

Undifferentiated hESC colonies were grown in 2-well chamber slides, fixed in freshly prepared 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100 (Sigma) in PBS. The nonspecific binding sites were blocked with 1% BSA in PBS. The cells were then incubated overnight at 4 °C with primary antibodies (OCT3/4; Santa Cruz Biotechnology 1:100, NANOG; 1:100, SSEA4; 1:40, TRA-1-60; 1:40, TRA-1-81; 1:40, Chemicon). After three washings with PBS, cells were incubated with appropriate FITC-conjugated secondary antibody for 1 h. For staining serial sections of embryoid bodies, the slides were dewaxed and permeabilized with Triton X-100. The preparations were blocked with 10% goat serum for 1 h followed by incubation with the primary antibody in a humidified chamber for 2 h. The following antibodies were analyzed: Nestin (Chemicon, 1:50), GATA4 (Chemicon, 1:50), and HNF4a (Chemicon, 1:50). Cells were washed with PBS containing 0.05% Tween 20 and incubated for 30 min at 37 °C with the secondary antibody, anti-mouse Alexa 568 or anti-rabbit Alexa 488 (Invitrogen, CA, USA, 1:500). Nuclei were counterstained with DAPI. The cells were observed under a fluorescence microscope (Nikon Eclipse E600, Kanagawa, Japan) to evaluate the stained areas.

Histological staining for alkaline phosphatase was carried out using a commercially available kit (Chemicon) following the manufacture's instructions.

For flow cytometric analysis, hESCs were first mechanically separated from the feeder layer followed by single cell dissociation using TrypLE Select (Invitrogen) enzyme. The cells were then stained with phycoerythrin (PE)-labeled OCT3/4 and SSEA-4 (BD Pharmingen) along with the respective isotype control. For staining with OCT3/4, the cells were first fixed and permeabilized in Cytotfix/Cytoperm Fixation/Permeabilization Solution (BD). Cells were analyzed by a FACScaliber Flow Cytometer (BD Biosciences, San Jose, CA). Ten thousand events were acquired and analyzed by CellQuest software.

Real-time PCR

Total RNA was isolated using the RNeasy mini kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's recommendations followed by cDNA synthesis using superscript reverse transcriptase (Invitrogen). For real-time PCR, approximately 50 ng of cDNA template was mixed with 2X PCR mix (Applied Biosystems, Foster City, CA) and diluted to 1X with 100 nM primers and water. Predesigned Assay on Demand TaqMan probes and primers were obtained from Applied Biosystems (ABI). Quantitative RT-PCR was performed on an ABI iCycler. PCR analyses were conducted in triplicates for each sample and the results were analyzed using qbase software (Sequence Detection Software Ver 1.2.2, 7500 Systems, ABI). Relative quantitation of gene

expression between multiple samples was achieved by normalization against endogenous 18 S ribosomal RNA (ABI) by using the ΔCt method of quantitation. Fold changes were calculated as $2^{-\Delta\Delta\text{Ct}}$.

Microarray analysis

The cells were collected at a low passage for the Relicell[®]hESC lines (Relicell[®]hES2, p24, and Relicell[®]hES3, p29) as well as from the previously characterized hESC lines, Relicell[®]hES1 (p30) and BG01 (p29). The hESC colonies with typical undifferentiated morphology were selected carefully and manually separated and collected so as to avoid any MEF contamination. Total RNA from each sample was isolated using the RNeasy mini kit (Qiagen, USA). The RNA samples were first qualified for microarray studies using a bioanalyzer (2100 Bioanalyzer, Agilent Technologies, CA, USA). Five hundred (500) ng of total RNA was used as a starting material for the microarray sample preparation. The sample preparation was carried out according to Illumina guidelines using an Ambion kit (Ambion Inc., TX, USA). A total of 750 ng purified cRNA labeled with UTP-biotin was used for hybridization and stained with Cy3. Samples were added in duplicates and processed using Illumina Genome Studio software. Data analysis was carried out using the gene expression analysis approach. The data were analyzed for functional clustering using an online software tool DAVID (<http://david.abcc.ncifcrf.gov/home.jsp>).

Karyotyping

Karyotype analysis was performed using standard methods of colcemid arrest and G-banding techniques. The hESCs were treated with colcemid (2 $\mu\text{g}/\text{ml}$) for 4 h followed by dissociation with prewarmed TrypLE Select. After neutralization with ES medium, the cells were resuspended in hypotonic KCl solution (0.0375 M) for 25 min. The cells were then fixed with Carnoy's fixative (glacial acetic acid:methanol; 3:1) and dropped onto wet precleaned slides. Chromosome spreads were Giemsa-banded and photographed using an Olympus BX40 microscope, and images were captured using a cyto vision digital imaging system.

HLA typing and STR genotyping

HLA profiling and STR genotyping were performed at GlobalStem Inc. (Rockville, MD, USA). HLA typing was done to determine the alleles present in each HLA locus on chromosome 6 using sequence-specific primers. The HLA –A, B, C, DR, and DQ alleles were analyzed.

STR profiling was done by multiplex PCR to simultaneously amplify the amelogenin gene and 15 highly polymorphic markers in the human genome. Loci analyzed included D8S1179, D21S11, D7S820, CSF1P0, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, and FGA.

In vitro differentiation potential

To assess the *in vitro* differentiation potential, hESC colonies from confluent cultures were manually cut into clumps of approximately 100–150 cells. These clumps were then cultured in low attachment bacteriological dishes (Nunc) to induce embryoid body formation. EB culture media were composed of 79% DMEM/F12 (Invitrogen), 20% SR (Invitrogen), 1% nonessential amino acid solution (Invitrogen), 1 mM glutamine (Invitrogen), and 0.1% β -mercaptoethanol (Sigma). Samples were collected at different time points: – initiation of EB formation (undifferentiated hESC; Day 0) and EB cultures on Days 5, 10, 15, and 20 and RNA was extracted from these samples for each time point. The expression profiles of the various lineage markers were determined in these samples by qRT-PCR for both hESC lines.

Expression of the lineage markers at the cellular level was determined in EBs by culturing them in suspension for 30 days followed by immunostaining of the serial sections with antibodies specific for ectoderm (Nestin), mesoderm (GATA4), and endoderm (HNF4a). The details are provided above.

In vivo differentiation potential

Similar to our earlier reported Relicell[®]hES1 (Pal et al., 2007); Relicell[®]hES2 and Relicell[®]hES3 were assessed for pluripotency *in vivo* by teratoma formation in SCID mice. Approximately, 7×10^6 undifferentiated cells were injected intramuscularly as clumps into four SCID mice (C.B-17) for each cell line. The mice which developed teratoma were sacrificed after 8–12 weeks and tumors were excised and fixed in 4% paraformaldehyde (Sigma) and H and E staining was performed for histological analysis.

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Appendix A. Supplementary data

Supplementary Fig. 1. Derivation of Relicell[®]hES2 and Relicell[®]hES3 hESC lines.

Supplementary Fig. 2A. Status of pluripotency markers as selected from the ISCI report in Relicell[®]hESC lines.

Supplementary Fig. 2B. Status of differentiated markers as selected from the ISCI report in Relicell[®]hESC lines.

Supplementary Fig. 2C. Heat map depicting the genes selected from the ISCI list and expressed by Relicell[®]hESC.

Supplementary Fig. 3. Karyotype analysis of Relicell[®]hESC lines.

Supplementary Table 1. Absence of twelve undifferentiated genes in Relicell[®]hESC cell lines.

Supplementary Table 2. Signaling pathway analysis.

Supplementary Table 3. HLA typing of the two Relicell[®]hESC lines.

Supplementary Table 4. STR profiles of Relicell[®]hES2 and Relicell[®]hES3 cell lines establish their unique identity.

Note: Supplementary materials related to this article can be found online at doi:10.1016/j.scr.2010.07.001.

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